Microgravity Cultivation of Cells and Tissues

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ABSTRACT

In vitro studies of cells and tissues in microgravity, either simulated by cultivation conditions on earth or actual, during spaceflight, are expected to help identify mechanisms underlying gravity sensing and transduction in biological organisms. In this paper, we review rotating bioreactor studies of engineered skeletal and cardiovascular tissues carried out in unit gravity, a four month long cartilage tissue engineering study carried out aboard the Mir Space Station, and the ongoing laboratory development and testing of a system for cell and tissue cultivation aboard the International Space Station.

INTRODUCTION

Exposure to microgravity can affect cells, tissues and the whole organisms at a variety of levels (Churchill, 1997, Nicogossian et al., 1994). Significant loss of bone mass, muscle strength, and cardiovascular fitness occur even when astronauts exercise regularly (Churchill, 1997), but the mechanisms underlying these physiological changes are not yet understood. Microgravity also changes growth patterns and carbohydrate processing in plants (Tripathy et al., 1996), and the behaviors of aquatic organisms (de Jong et al., 1996). Biological experiments carried out in microgravity could potentially improve our understanding of the basic mechanisms underlying the above effects. In particular, controlled in vitro studies of cells and tissues can be designed to distinguish the specific effect of reduced gravity from the complex milieu of factors involved in spaceflight (i.e. launch, landing, radiation). Microbes (e.g. yeast) can provide genetic models for studies of molecular responses to microgravity, while motile aquatic microorganisms (e.g. Euglena) can help elucidate mechanisms of gravitaxis, chemotaxis, and phototaxis. Mammalian tissue culture studies can improve our understanding of the human responses to pseudo-weightlessness (e.g. during embryogenesis or prolonged immobilization) and expedite the development of countermeasures to prevent astronaut deconditioning during prolonged missions (e.g. on a space station or to Mars).

Microgravity studies refer herein to two different experimental situations: ground-based studies in which the biological specimens are cultured in continuous free-fall (simulated microgravity), and space studies (actual microgravity). The combination of ground and space studies provides a unique opportunity to identify biological mechanisms of gravity sensing, transduction, and response (e.g. cell migration, tissue development), as well as secondary effects of gravity on mass transfer in the cellular microenvironment. In this paper, we review (1) tissue engineering studies carried out in rotating bioreactors on earth, (2) a space study of tissue engineered cartilage carried out in a rotating bioreactor aboard the Mir Space Station, and (3) the ongoing design and testing of complementary new flight hardware for use aboard the International Space Station (ISS).

TISSUE ENGINEERING STUDIES IN ROTATING BIOREACTORS ON EARTH

The rotating bioreactor is a tissue culture vessel that has been used in ground-based studies of a wide variety of normal and transformed cell types (Unsworth and Lelkes, 1998; Freed et al., 1998). The equipment was developed at NASA's Johnson Space Center (JSC) (Schwarz et al., 1992) and is commercially available from Synthecon (Houston, TX). One design is the Slow Turning Lateral Vessel (STLV) which is configured as the 110 mL annular space between two concentric cylinders, which rotate in a horizontal plane about their common axis as a solid body (Fig. 1a). Viscous coupling induces a rotational flow field that can suspend large 3D tissues (e.g., 5-7 mm diameter x 2-3 mm thick) without external fixation or mechanical stirring (Freed and Vunjak-Novakovic, 1995). In particular, Vessel rotational speed is adjusted such that tissues remain suspended close to a stationary point within the Vessel, relative to an observer on the ground, due to a dynamic equilibrium between the acting gravitational, centrifugal and drag forces (F_g , F_c and F_d , respectively) (Fig. 1b). The condition that a tissue is cultured freely falling on Earth simulates some aspects of microgravity.

Fluid-dynamic experiments were carried out using rotating bioreactors containing 3-dimensional (3D) constructs. Hydrodynamic conditions were laminar, mixing in the bulk phase was very efficient, and shear stresses were below 1 dyn/cm², as assessed by particle image velocimetry (i.e. cross-correlation of particle displacement fields in a rotating vessel containing a construct and microparticulate tracers for the liquid phase) (Neitzel et al., 1998). Rotating vessels can thus provide high rates of mass transfer to growing tissues while maintaining a low shear environment during cultivation.

We have carried out a variety of studies using an in vitro system with three components: isolated cells, polymeric templates, and tissue culture bioreactors (Fig. 2). In particular, cells (i.e. chondrocytes, bone marrow stromal cells, BMSC, and cardiac myocytes respectively isolated from cartilage, bone marrow, and cardiac tissue) were cultured on 3D synthetic, biodegradable polymer scaffolds (i.e. meshes of polyglycolic acid, PGA, or sponges of polylactic co-glycolic acid, PLGA) in rotating bioreactors. The chondrocytes formed engineered cartilage containing glycosaminoglycan (GAG) and collagen type II (Freed et al., 1998) that responded to compressive loads in a manner characteristic of native cartilage (Freed et al., 1997, Vunjak-Novakovic et al., 1999). The BMSC formed engineered bone that contained mineralized extracellular matrix (ECM), collagen type X, osteopontin and bone sialoprotein (Martin et al., 1998, 1999). The cardiac myocytes formed cardiac muscle that expressed specific

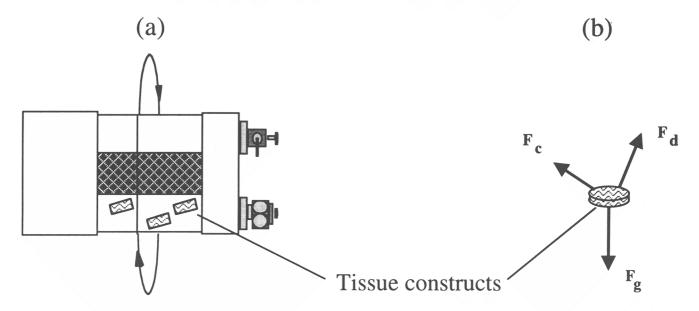


Figure 1. A rotating bioreactor. The STLV is a 110 mL vessel that houses approx 12 tissue constructs, each 5-10 mm diameter x 2-6 mm thick. (b) By adjusting the vessel rotation speed (e.g. 12 - 40 rpm), the constructs can be maintained in a state of continual free-fall due to a dynamic equilibrium between the acting forces (gravitational F_g , centrifugal F_c , and drag F_d).

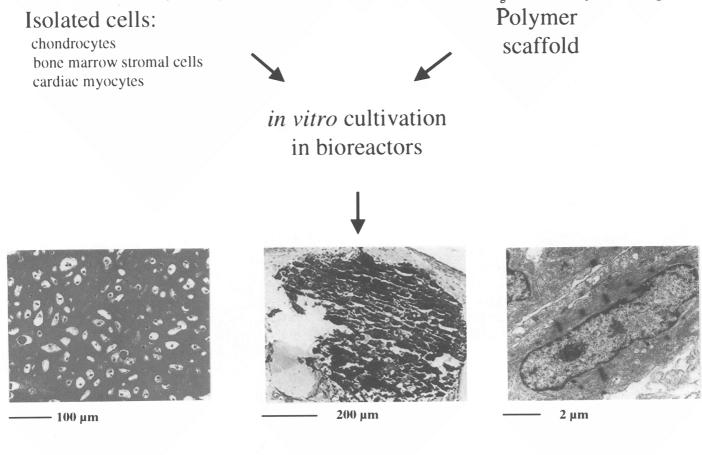


Figure 2: Model system. Isolated cells (e.g. chondrocytes, bone marrow stromal cells, or cardiac myocytes) are seeded onto polymer scaffolds and cultured in rotating bioreactors. The photographs show representative examples of the resulting engineered cartilage, bone and cardiac muscle (light and transmission electron micrographs).

Bone

Cardiac muscle

Cartilage

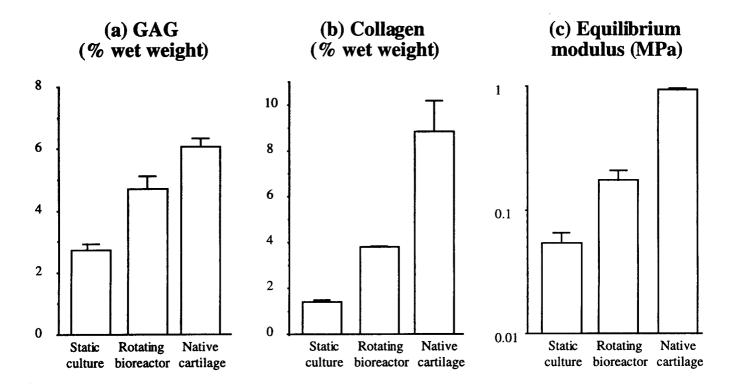


Figure 3: Rotating bioreactors improve the composition and function of engineered cartilage. Chondrocyte-PGA constructs cultured for 6 weeks in rotating bioreactors were compared to otherwise identical constructs cultured statically for 6 weeks and to freshly explanted native calf cartilage with respect to (a, b) wet weight fractions of GAG and collagen, and (c) equilibrium modulus (i.e. mechanical stiffness). Data are the Average \pm SD of 3-6 independent measurements.

proteins (cardiac myosin and troponin-T, sarcomeric tropomyosin), contained sarcomeres and intercalated discs, and contracted both spontaneously and in response to electrical stimulation (Freed and Vunjak-Novakovic, 1998, Bursac et al., 1998, Carrier et al., 1998).

As compared to conventional tissue culture methods, rotating bioreactors provided a significantly better environment for cartilage tissue engineering (Vunjak-Novakovic et al., 1999). Constructs based on bovine calf chondrocytes and PGA scaffolds cultured for 6 weeks in rotating bioreactors had significantly higher fractions of cartilaginous ECM (i.e. GAG) and superior mechanical properties (i.e. equilibrium modulus) compared to statically grown constructs (Fig. 3), but remained inferior to native calf cartilage. It is possible that the mechanism of enhanced chondrogenesis in rotating vessels (e.g. hydrodynamic fluctuations in shear and pressure) was analogous to that previously reported for dynamic mechanical compression (Buschmann et al, 1995) and intermittent hydrostatic pressure (Carver and Heath, 1998).

TISSUE ENGINEERING OF CARTILAGE ABOARD MIR

For the first long-term study of tissue culture in space, engineered cartilage was selected as a model musculoskeletal tissue because of its resilience and low

metabolic requirements (Freed et al., 1997). Constructs based on bovine calf chondrocytes and PGA scaffolds were cultured for three months in rotating biroeactors on earth and then transferred to flight hardware (the Biotechnology System, BTS) and cultivated for an additional four months either on the Mir Space Station or on earth. Specifically, one BTS containing ten constructs was transferred to Mir's Priroda module via the US space shuttle STS-79 (9/16/96 launch), operated in microgravity, and brought back to earth via STS-81 (1/22/97 landing). A second BTS with ten constructs was operated at NASA-JSC to serve as a control at unit gravity.

The Biotechnology System (BTS) is one component of the ISS Biotechnology Facility (BTF) being developed by NASA-JSC (NASA web site). The BTS has approximate dimensions of 17 x 20 x 25 inches (2 1/2 middeck Shuttle lockers) and weighs 137 lbs. Tissue constructs were cultured in an 125 mL bioreactor configured as the annular space between two cylinders, the inner of which was covered with a nylon mesh and had a disc attached at one end which served as a viscous pump (Fig. 4). The bioreactor was connected to a recirculation loop consisting of a silicone membrane gas exchanger (Avecor model 0400-2A, Plymouth, MN) and a peristaltic pump (Randolph model 250, Manchaca, TX). The BTS was maintained at 37°C and had access ports for infusing

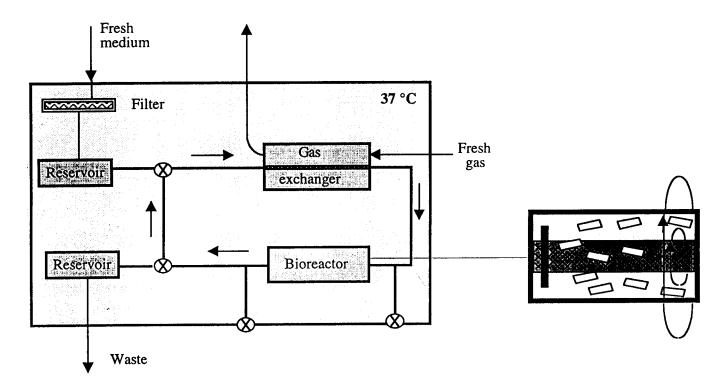


Figure 4: The Biotechnology System (BTS). The BTS is flight hardware consisting of a 125 mL volume rotating, perfused vessel that can house approximately 10 tissue constructs each measuring 5-9 mm diameter x 4-8 mm thick. Medium recirculation (i.e. between the bioreactor and gas exchanger), medium infusion (i.e. from a reservoir), and rotation speeds of the inner and outer cylinders are preset and computer-controlled in-flight.

fresh media and gas (10% CO₂ in air), sampling culture media, and removing waste media (Fig. 4).

Concentration gradients within the bioreactor were minimized by mixing induced either by differential rotation of the inner and outer cylinders at 10 and 1 rpm, respectively (in microgravity) or by gravitational construct settling during solid body rotation of the bioreactor at 28 rpm (at unit gravity) (Freed et al., 1997). Medium was recirculated between the bioreactor and the gas exchanger at 4 mL/min for 20 min four times per day, and approximately 50 to 100 mL of fresh medium were infused into the system once per day. Under these conditions, metabolic parameters were maintained within previously established target ranges (e.g. pH between 6.9 and 7.4; dissolved oxygen concentrations between 71 and 127 mmHg, as assessed in space using a portable cartridge) (G3+, I-Stat, Princeton, NJ). Constructs were assessed at the time of launch and after four additional months of cultivation on either Mir or earth, and compared to natural calf articular cartilage.

Constructs grown on Mir tended to become more spherical while those grown on earth maintained their initial discoid shape, as assessed histologically (Fig. 5a) and from the respective aspect ratios. These findings could be related to differences in cultivation conditions. In particular, videotapes showed that constructs floated freely in microgravity but settled and collided with the rotating

vessel wall at unit gravity. On Mir the constructs were exposed to uniform shear and mass transfer at all surfaces such that the tissue grew equally in all directions, while on earth the settling of discoid constructs tended to align their flat circular areas perpendicular to the direction of motion, which increased shear and mass transfer circumferentially such that the tissue grew preferentially in the radial direction. Constructs from both groups appeared continuously cartilaginous over their entire cross-sections (4-8 mm), with the exception of fibrous outer capsules (0.15 - 0.45 mm thick). Cells isolated from post-flight tissues were alive and metabolically active, as demonstrated by their ability to attach to Petri dishes, proliferate, and enzymatically convert an unlabeled substrate into a fluorescent dye (Fig. 5b). Mir-grown constructs consisted of 95-99% viable cells as assessed by trypan blue exclusion, and incorporated radiolabeled tracers into macromolecules 30 h post-flight at rates comparable to those measured for earth-grown constructs.

At the time of launch and after additional cultivation on Mir and on earth, constructs contained 13, 14, and 19 million cells, respectively (Freed et al., 1997). On earth, construct wet weights increased 1.7-fold between three and seven months, which could be attributed to increasing amounts of cartilage-specific tissue components (i.e. collagen type II and GAG) (Fig. 6 a&b). On Mir, in contrast, construct wet weights increased by 1.3-fold over

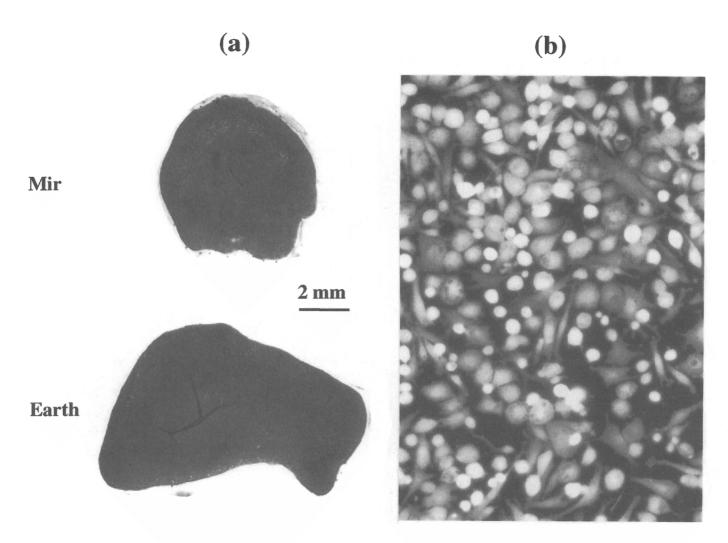


Figure 5: Cell and tissue growth in space. (a) full histological cross-sections of constructs grown on Mir and on earth, stained for glycosaminoglycan, (b) cells isolated from a Mir-grown construct after 2 days of monolayer culture; stained cells are expressing intracellular esterase.

the same time interval, due to deposition of collagen and unspecified components (Fig. 6 a&b). The fraction of the total collagen that was type II decreased, but not significantly between the time of lauch and landing, demonstrating relatively good maintenance of the chondrocyte phenotype (Freed et al., 1997). The equilibrium modulus of earth-grown constructs was comparable to that of native calf cartilage and was 3-fold higher than that of Mir-grown constructs, as assessed in radially confined compression (Fig. 6c).

The finding that constructs in the earth group had markedly higher wet weights, GAG fractions, and compressive moduli than constructs in the Mir group might be attributed to differences in cultivation conditions (i.e. free floating vs. gravity settling). In particular, spherical shape, relatively low GAG fraction and inferior mechanical properties of Mir-grown constructs might be attributed to reduced physical forces in the microgravity environment of

space. However, we cannot distinguish between the relative contributions of microgravity, launch, landing, and local environmental factors (e.g. cosmic radiation), and there is a need for further control studies to be carried out in artificial gravity in the space environment.

NEW FLIGHT HARDWARE FOR THE ISS

The Cell Culture Unit (CCU) is flight hardware being developed by Payload Systems Inc. in collaboration with M.I.T. and NASA's Ames Research Center (ARC) for use aboard the ISS (Searby et al., 1998). The CCU has dimensions of 18.5 x 21 x 21 inches (2 Shuttle middeck lockers), weighs 147 lbs, and complements the BTS with respect to several design features as described below.

The CCU accommodates diverse specimens (animal, microbial and plant cells, motile aquatic microorganisms, and tissues less than 4 mm in diameter) and be operable both in microgravity (i.e. in the ISS habitat

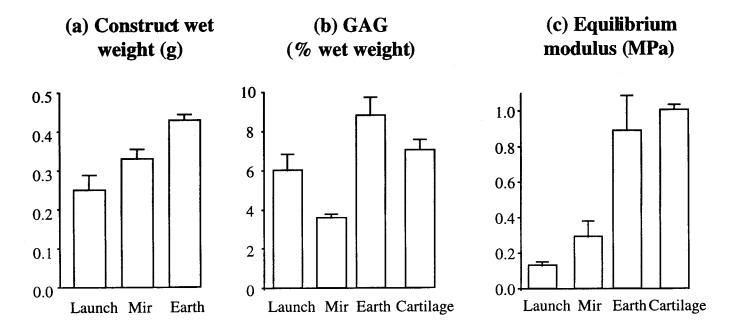


Figure 6: Spaceflight affects tissue structure and function. Cartilaginous constructs were cultured for 3 months in rotating bioreactors on earth prior to launch are compared to constructs cultured for an additional 4 months on Mir or on earth, and to native calf cartilage with respect to (a) wet weight, (b) GAG as a % of wet weight, and (c) equilibrium modulus. Data are the Average ±SD of 2-7 independent measurements.

holding rack) and in artificial gravity (i.e. in the ISS centrifuge). Biological specimens are housed in cell specimen chambers (CSCs, numbering 8, 12 or 24 with respective volumes of 30, 10 or 3 mL). Each CSC is connected to a recirculation loop consisting of a miniature peristaltic pump (model P625/66/NC Instech, Plymouth Meeting, PA), a silicone tubing gas exchanger, and reservoirs for fresh media, additives (e.g. growth factors, fixatives), and waste (Fig. 7). Medium enters the central compartment of the CSC (the cell culture space) and exits through a porous membrane. The bottom surface of the CSC is made of optical quality glass coated with magnesium fluoride for video observation. Individual CSCs and their recirculation loops are grouped in four independently controlled quadrants and mounted on a circular tray that can be rotated. An automated sampling system allows for collection and storage of up to 60 samples of cells or cell-free media per experiment. The system can be maintained at a range of temperatures (4-45°C) and provides light at a variety of wavelengths (400-730 nm) and intensities (10-30 μ mol/m²s). Each CSC and its loop can be isolated from the remainder of the system if and when needed (e.g. in the event of contamination or after fixation of the CSC contents). CSC loops are also compatible with the compound microscope in the ISS life sciences glovebox.

The small scale of this design and the need to maintain physiological conditions in multiple CSC loops instigated the development of reliable optical biosensors

based on the principles of fluorescence lifetime frequency modulation (Szmacinski and Lakowicz, 1995). In particular, a fluorphore sensitive to oxygen or pH is immobilized in a small patch and placed in the path of flowing medium, excited with light using a light emitting diode (LED), and the phase angle between the emission and excitation is measured using a photomultiplier tube (Fig. 8a). In this system the output is, within certain limits, independent of both the intensity of excitation light and the amount of fluorophore, minimizing the need for recalibration. In one prototype, a fluorophore (a complex of ruthenium) was immobilized within a small silicone patch and used to monitor medium oxygen concentrations for up to 1 month. A representative calibration curve obtained for cell culture medium at 37°C is shown in Fig. 8b. A stable correlation was observed between the phase angle measured using the biosensor and the medium partial pressure of oxygen measured using a standard gas-blood analyzer. The same approach is was recently used to monitor medium pH and can potentially be extended to glucose and various immunomolecules.

Individual CCU components are now undergoing preliminary testing using biological specimens that were selected to represent the variety of samples and the ranges of experimental conditions to be studied aboard the ISS. The specimens included monolayers of myoblasts (the C2C12 cell line) and osteoblasts (derived from bone marrow stroma), 3D skeletal muscle organoids (based on embyonic chick skeletal myoblasts), and suspension

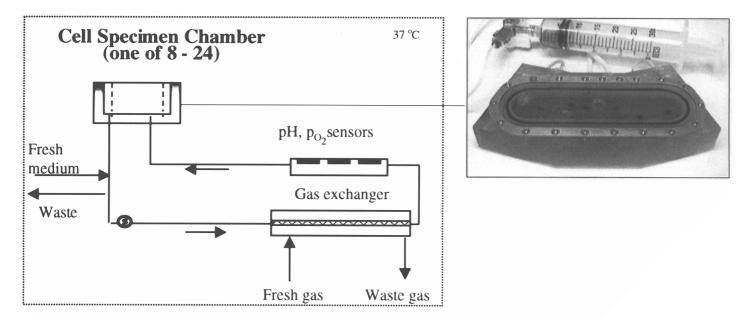


Figure 7: The Cell Culture Unit (CCU). The CCU is flight hardware currently under development that consists of 8-24 Cell Specimen Chambers (CSCs) that can house a variety of biological specimens. CSCs are connected to a gas exchangers by an individual recirculation loops and to reservoirs of fresh and waste media.

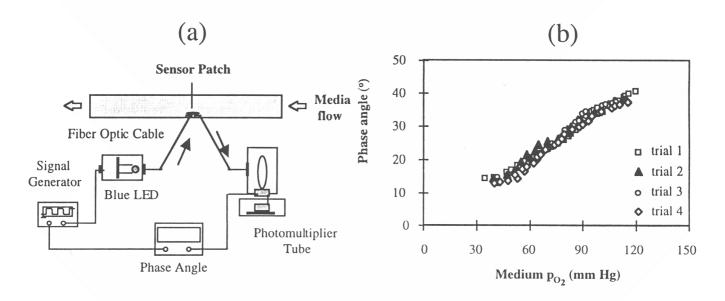
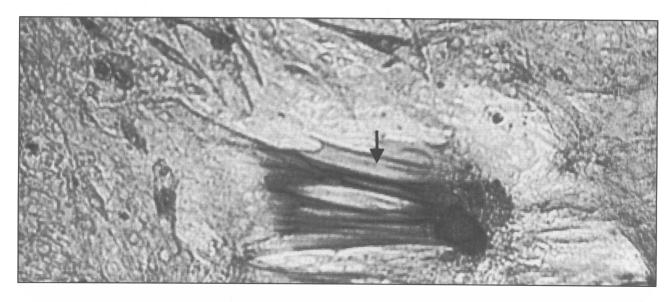


Figure 8: Oxygen sensor (a) working principle **(b)** Correlation between dissolved oxygen concentration and phase angle measured in medium with 10% serum as observed in 4 sequential trials carried out over a period of 1 month without recalibration.

cultures of yeast (*Saccharomyces cerevisiae*) and an aquatic microorganism (*Euglena gracilis*).

Murine C2C12 myoblasts and bovine bone marrow stromal cells (BMSC) were tested in monolayer cultures in perfused CSCs operated at 37°C for 3-4 weeks as follows. C2C12 cells were plated on laminin- and collagen-coated glass coverslips (3 x 10⁴ cells/cm² in

medium containing 20% serum) and allowed to attach for 24 h in a 5% $\rm CO_2$ incubator. One cell-seeded coverslip was then transferred into the 3 mL CSC and perfusion was established at 0.5 mL/min. After 5-6 days, the cells were confluent and medium serum concentration was reduced to 2% to promote differentiation. Within 10 days, the cells fused into myotubes that expressed the muscle-specific



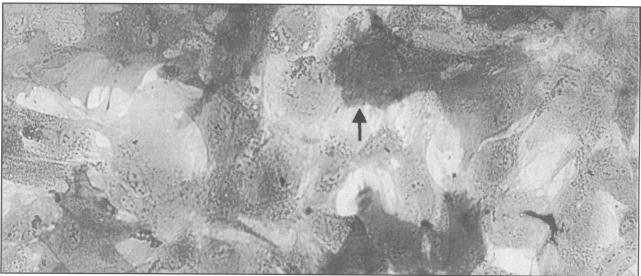


Figure 9: Monolayer cultures (a) 10 day culture of murine C2C12 cells. Arrow denotes a myotube stained for tropomyosin (b) 13 day culture of bovine bone marrow stromal cells. Arrow denotes a cell expressing alkaline phosphatase activity.

protein tropomyosin (**Fig. 9a**). BMSC were likewise plated on glass coverslips (5 x 10^3 cells/cm²in medium containing 10% serum, $50~\mu g/mL$ ascorbic acid, and 1~ng/mL fibroblast growth factor-2, FGF) and allowed to attach for 24 h. Seven cell-seeded coverslips were transferred into the 30 mL CSC and perfusion was established at 1~mL/min. After 5-6 days, the cells were confluent and the FGF was replaced with betaglycerophosphate (7 mM) and dexamethasone (10~nM) to promote differentiation. Within 13~days, BMSC underwent osteogenic differentiation, as assessed by alkaline phosphatase expression (**Fig. 9b**) and the formation of a mineralized ECM, as assessed by alizarin red staining (data not shown). Recently, a 4 week culture of 3D muscle organoids (approximately 4 mm

diameter and 30 mm long) was carried out in the 30 mL CSC perfused at 3 mL/min (data not shown).

The yeast Saccharomyces cerevisiae and the microorganism Euglena gracilis were tested in suspension cultures in perfused CSCs operated at 23°C for between 4 days and 4 weeks as follows. Saccharomyces, which has a high metabolic rate and a rapid doubling time, was tested in a system consisting of a gas permeable reservoir bag (4R2110, Baxter Healthcare, Deerfield, IL) a gas exchanger (a coil of silicone tubing 1 m long with 1/16 inch ID x 1/32 inch wall, Cole Parmer, Vernon Hills, IL), the pump, and a 30 mL CSC. The CSC was inoculated with 5 x10⁴ cells/mL and perfused at 15 mL/min using 3 inlets; the direction of flow was periodically reversed in an

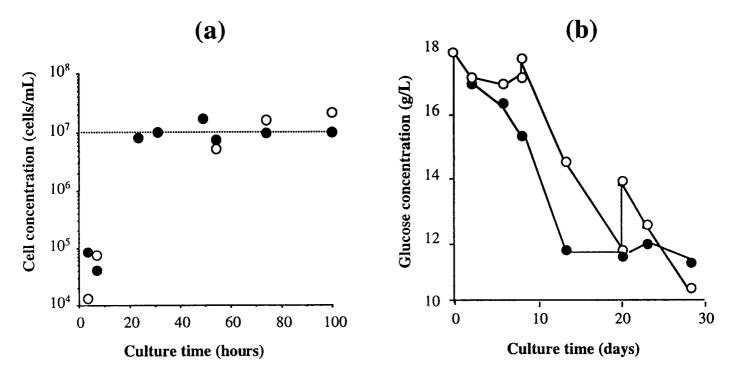


Figure 10: Suspension cultures (a) Saccharomyces cerevisiae and (b) Euglena gracilis cultured in CSCs (open symbols) or shake-flasks (closed symbols). Data points represent individual measurements.

attempt to reduce membrane fouling. An additional culture was carried out in a 250 mL shake-flask operated at 250 rpm and 30 °C. Cells were cultured in medium containing glycerol instead of dextrose in an attempt to reduce their metabolic activity. The final cell density obtained in the CSC at 23 °C (approximately 10⁷ cells/mL) was comparable to that achieved in the shake flask maintained under optimal conditions (30 °C) (**Fig. 10a**). However, the duration of these studies was limited to 4 days, after which perfusion was blocked by gas bubbles (presumably due to cellular production of CO₂) and fouling of the CSC membrane (presumably due to accumulation). Our ongoing efforts include modifying the flow regime, gas exchanger, membrane characteristics, and medium composition.

Euglena, a motile aquatic microorganism, was tested in a system consisting of the reservoir bag, the pump, and a 30 mL CSC. The CSC was inoculated with 10⁴ cells/mL and perfused through one inlet at 3 mL/min. An additional culture was carried out in a 125 mL shakeflask (120 rpm, 23°C). Cells were cultured in medium containing glucose to eliminate their light requirement. Cellular utilization of glucose was assessed over 4 weeks of culture (Fig. 10b); CSC medium replacements were responsible for the increases in glucose concentration on culture days 8 and 20. Comparable cell motility, aggregation and sedimentation were observed in the CSC and the shake flask. However, cells were found to have moved from the CSC into the recirculation loop; ongoing efforts to solve this problem include the use of a filter at the CSC inlet.

CONCLUSIONS

Microgravity studies have been carried out both on earth (in simulated microgravity) and in space (in actual microgravity), using a variety of cells and tissues. In ground studies, rotating bioreactors provided a favorable environment for engineering 3D skeletal and cardiovascular tissues, presumably due to the combination of efficient mass transfer and dynamic laminar flow at tissue surfaces. In a four month long ground-controlled study of engineered cartilage in flight-qualified rotating bioreactors, the cells maintained viability and differentiated phenotype. Tissues grown on earth had markedly higher wet weights, GAG fractions, and compressive moduli than those grown on Mir, presumably due to the reduction of physical forces in the microgravity environment of space. Complementary flight hardware under development includes cell culture units to be operated both in microgravity and in artificial gravity (in the ISS centrifuge), in an attempt to distinguish specific effects of gravity from other factors (e.g. launch, landing, radiation) present during spaceflight. Prototype hardware has been tested using cells in monolayers, suspensions and 3D cultures, and optical sensors are being developed for on-line monitoring of pH and oxygen culture medium. Further microgravity studies of cell and tissues similar to those described in this paper are expected to help identify mechanisms underlying gravity sensing and transduction in biological organisms.

Acknowledgments

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